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Our goal is to establish the molecular determinants that account for the pore properties of ligand-regulated channel properties. We are modeling hypothesized pore forming structures from acetylcholine receptors using semi-empirical potential energy functions. The M2δ segment of the nicotinic acetylcholine receptor from Torpedo californica is a candidate for the channel lining structure. It forms ionic channels in human erythocyte membranes and in lipid bilayers. Furthermore, we designed and synthesized a tethered tetramer containing four M2δ-oligopeptides. The complete 101 residue protein forms channels in lipid bilayers with properties that resemble those of authentic cholinergic receptors. The general validity of this approach is currently being assessed.

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RESEARCH OBJECTIVE: Our goal is to establish the molecular determinant of the pore properties in ligand-regulated channel proteins.

PROGRESS (Year 1):

1. Molecular modeling of the pore forming structures of ligand-gated channel proteins.

We pursued the modeling of the pore forming structures of two channel proteins with different primary structures and oligomeric number; namely, the voltage sensitive sodium channel and the nicotinic cholinergic receptor. Low-energy arrangements of α-helical bundles were calculated by semi-empirical potential energy functions and optimization routines and were further refined using molecular dynamics. The ion-conducting pore is considered to be a symmetric or pseudosymmetric homooligomer of 3-5 amphipathic α-helices arranged such that the polar residues line a central hydrophilic pathway and the apolar residues face the hydrophobic bilayer interior. The channel lining exposes either charged (Asp, Glu, Arg, Lys) or polar-neutral (Ser, Thr) residues. A bundle of 4 parallel helices constrained to C_4 symmetry, the helix axis aligned with the symmetry axis, and the helices constrained to idealized dihedral angles, produces a structure with a pore of the size inferred for the sodium channel protein (area ~ 16 $Å^2$). Similarly, a pentameric array optimized with constraints to maintain C_5 symmetry and backbone torsions characteristic of α-helices adopts a structure that appears well suited to form the lining of the nicotinic cholinergic receptor (pore area ~ 46 $Å^2$). Thus, bundles of amphipathic α-helices satisfy the structural, energetic, and dynamic requirements to be the molecular structural motif underlying the function of ionic channels.

2. The M2δ transmembrane domain of the nicotinic cholinergic receptor forms ion channels in human erythrocyte membranes.

We examined the notion that a synthetic peptide with the sequence of the M2δ segment of the nicotinic acetylcholine receptor from *Torpedo californica* forms ionic channels in biological membranes. For this purpose we selected human erythrocyte membranes and assayed channel formation by determining both hemoglobin and K^+ release. Indeed, this peptide forms a permeability pathway with an apparent cross-sectional diameter of 7-9 Å. The M2δ pore is oligomeric and a pentamer is the species that accounts for the properties of the permeation path. Peptides that mimic other identifiable segments of the *Torpedo* acetylcholine receptor, M1δ and MIR, do not form channels in erythrocytes under the same conditions.

3. Synthesis of tetrameric synthetic channel proteins was achieved.

We implemented the design principles outlined by Mutter and synthesized tethered tetramers containing the channel-forming domains of the *Torpedo californica* acetylcholine receptor (AChR) δ subunit

transmembrane segment 2 (M2) attached to a carrier template. The carrier is a nine-aminoacid backbone with sequence K*KK*PGK*EK*G with K* containing N^{α}-N-*tert*-butyloxycarbonyl (tboc), N^{ε}-9-fluorenylmethoxycarbonyl (fmoc) to generate four branch points. Oligopeptides are then attached to template in a stepwise manner at the four base-deprotected lysine sidechains. A parallel array of the 4-oligopeptides is determined by tethering them to the carrier.

The complete 101 residue protein does indeed form channels in lipid bilayers which reproduce several features that are characteristic of authentic AChR channels, such as single channel conductance, cation selectivity, transitions between closed and open states in the millisecond time range. An analogue protein, in which the serine residue in position 8 is replaced for alanine in each of the four M2δ 23-mer peptides, also forms channels which, however, exhibit lower single channel conductance. By contrast, a similar tethered tetramer with M1δ-peptides does not form channels in accord with expectations. The general validity of this strategy to other channel sequences and oligomeric number is currently being explored. Thus, this novel class of synthetic channel proteins enriches our armamentarium directed towards the elucidation of structure-function relationships.

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THE M2δ TRANSMEMBRANE DOMAIN OF THE NICOTINIC CHOLINERGIC RECEPTOR FORMS ION CHANNELS IN HUMAN ERYTHROCYTE MEMBRANES

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SUMMARY. A synthetic peptide with the sequence of the M2δ segment of the nicotinic acetylcholine receptor from *Torpedo californica* forms pores in human erythrocyte membranes as determined by hemoglobin and potassium release. This peptide forms a permeability pathway with an apparent cross-sectional diameter of 7-9 Å. The M2δ pore is oligomeric and a pentamer is the species that accounts for the properties of the permeation path. Peptides that mimic other identifiable segments of the *Torpedo* acetylcholine receptor, M1δ and MIR, do not form channels in erythrocytes under the same conditions.

INTRODUCTION. The nicotinic acetylcholine receptor (AcChoR) of *Torpedo californica* is composed of four glycoprotein subunits (α, β, γ, δ) with stoichiometry $\alpha_2\beta\gamma\delta$ (1, 2). A high degree of amino acid sequence homology exists among the four subunits, and all exhibit four putative transmembrane regions designated as M1, M2, M3 and M4 (3). *In vivo*, the AcChoR pentamer acts as a ligand activated cation channel (4) with an effective pore diameter of ~ 7 Å (5). The specific assignment of subunits involved in channel lining has been a subject of intense investigation. Evidence suggests that M2 is the segment which lines the pore (4, 6-8). Significantly, a synthetic 23-mer peptide with the sequence of M2δ forms ion channels in lipid bilayers with single channel properties that emulate those of authentic AcChoR ion channels (8). Here we provide evidence of channel formation by the AcChoR M2δ peptide in biological membranes.

MATERIALS AND METHODS

Peptides. Peptides were synthesized by solid phase methods on an Applied Biosystems model 430 peptide synthesizer, purified by HPLC and sequenced, essentially as previously described (8, 9). The amino acid sequences of the peptides studied are: M2δ - EKMSTAISVLLAQAVFLLLTSQR (8), M1δ - LFYVINFITPCVLISFLASLAFY (8), MIR (a segment of the main immunogenic region) - VNQIVETNVR (10), and a peptide composed of 23 serine residues (poly S).

Hemolysis Assay. Hemolysis was assayed according to Tosteson et al. (11). Briefly, recently outdated blood from the Veteran's Administration Medical Center Blood Bank (La Jolla, CA) was washed three times with 0.3 M sucrose buffer (0.3 M sucrose, 0.01 M Tris-Hepes, 0.001 M EGTA,

The abbreviations used are: AcChoR, acetylcholine receptor, MIR, main immunogenic region, TFE, trifluoroethanol, Hb, hemoglobin.

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pH 7.4). An erythrocyte suspension was made 1% (v/v), and 2.5 ml samples were used for all assays. Synthetic peptides were dissolved in trifluoroethanol (TFE; Aldrich, Milwaukee, WI) and added to erythrocyte suspensions with immediate vortexing. Suspensions were incubated at 22 ± 2°C for desired time periods, whereupon two 1 ml aliquots were withdrawn and layered over 0.1 ml dibutylphthalate (Aldrich, Milwaukee, WI). Samples were immediately centrifuged for 5 minutes in an Eppendorf Model 5414 centrifuge, and the absorbance of the supernatants at 540 nm was recorded. The effect of equivalent amounts of pure TFE (5-30 µl) without peptide were subtracted from the lysis produced by peptides. Total lysis was obtained by solubilizing erythrocytes with Triton X-100 at a final concentration of 0.5% (v/v). K^+ was measured on a Perkin-Elmer Atomic Absorption Spectrophotometer Model 5000 (λ = 766.5 nm). Supernatants were combined, and 1 ml aliquots diluted to a final volume of 5 ml with distilled water.

RESULTS AND DISCUSSION

Synthetic M2δ peptide has hemolytic activity. Erythrocytes suspended in sucrose buffer are lysed by the synthetic M2δ peptide. Figure 1 illustrates the time course of hemoglobin (Hb) release produced by three different concentrations of M2δ. Initially, Hb release increases linearly with time, leveling off at a steady state value. K^+ release from erythrocytes is also a measure of cell lysis as illustrated in Figure 2B. The time courses of Hb and K^+ release are similar, although the initial rate of K^+ release is faster than that for Hb.

M2δ forms channels in lipid bilayers (8). Therefore, a likely mechanism of lysis is that M2δ creates a pore through which the high levels of intracellular K^+ exit the cell. Accordingly, K^+ efflux generates an osmotic imbalance leading to cell lysis. This model implies that external sucrose is too large to pass through the M2δ pore. Therefore, sucrose in the buffer was replaced by Tris (at 0.15 M), and no lysis was obtained (Figs. 1 and 2A). Tris was selected for this assay because it is known to permeate through both the authentic AcChoR (5) and the M2δ pore (8). Presumably, $Tris^+$ equilibrates with intracellular K^+, and no osmotic imbalance is created. The dimensions of $Tris^+$ (8Å x 7Å x 6Å) (12) and sucrose (11Å x 9Å x 8Å) (13) predict an effective cross-sectional diameter of the permeability path formed by the M2δ pore of 7-9 Å, in agreement with the apparent cut-off size of the synthetic M2δ (8) and authentic AcChoR (5) channels.

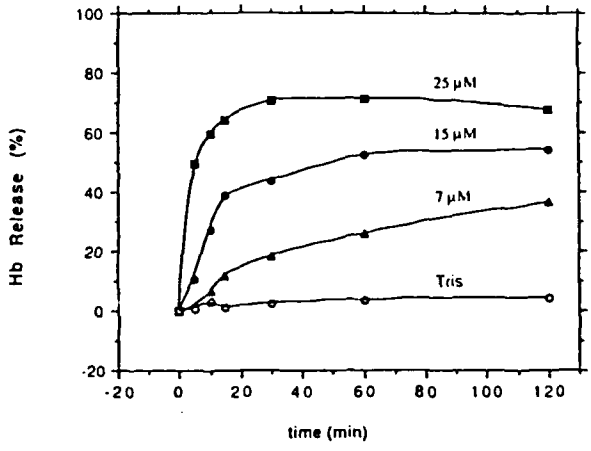

Fig. 1. Hb release from erythrocytes suspended in sucrose buffer supplemented with M2δ at 25 µM (■), 15 µM (●), and 7 µM (▲). Open circles (○) designate Hb release from erythrocytes suspended in 0.15 M Tris buffer containing M2δ (15 µM).

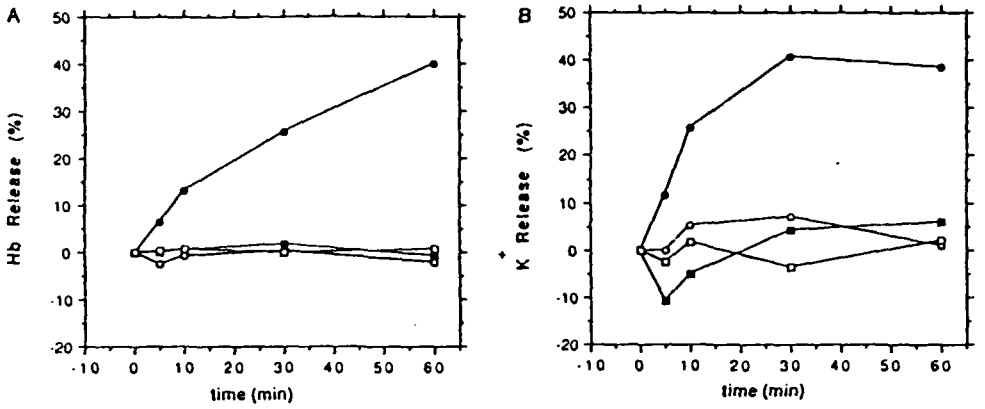

Fig. 2. Hb release (A) and K^+ release (B) from erythrocytes suspended in sucrose buffer (●, ■) or in Tris buffer (○, □) and exposed to 15 μM M2δ (●, ○) or 15 μM polyserine, n = 23 (■, □).

The M2δ pore is oligomeric. A plausible structural model for the ion-conductive pore formed by M2δ postulates a pentameric array of 5 amphipathic α-helices arranged such that the polar residues line a central hydrophilic pathway, and the apolar residues interact with the apolar core of the bilayer (8). Information about the size of oligomeric channels is obtainable from membrane conductance measurements in planar lipid bilayers (14). Double logarithmic plots of conductance vs. concentration of channel forming peptide have a slope equal to the number of peptides per channel. Since the rate of hemolysis is dependent on membrane conductance, a double logarithmic plot of the initial rate of hemolysis vs. concentration of M2δ will have a slope equal to the size of M2δ oligomers involved in the rate limiting step of the hemolysis pathway. Accordingly, Figure 3 shows that the assembly of a trimer is the rate limiting step in the formation of a functional ion channel. However, based on Tris permeability (5, 8), it is likely that pentamers are the dominant species responsible for the M2δ conductive pore.

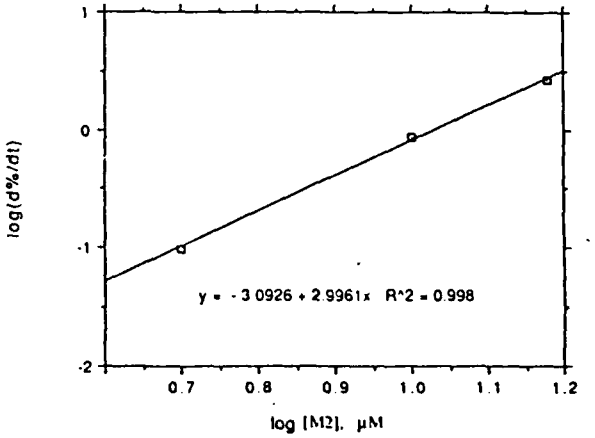

Fig. 3. Plot of log (initial rate of hemolysis) vs. log [M2δ]. Initial rates are initial slopes from a plot of % Hb release vs. time. Initial rates for 5, 10 and 15 μM M2δ were determined in duplicate, and the mean rates were plotted vs. [M2δ]. Slope of 3.0 was calculated by linear regression.

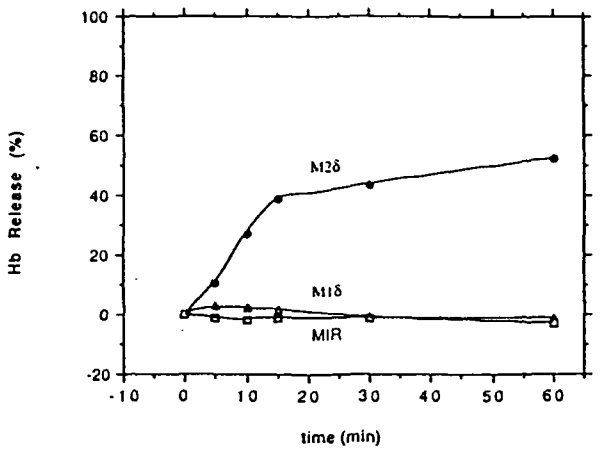

Fig. 4. Hb release from erythrocytes suspended in sucrose buffer containing 15 μM M2δ (●), M1δ (□), or MIR (Δ).

Specificity of the assay. To test the specificity of M2δ hemolytic activity, synthetic peptides with sequences derived from other AcChoR structural domains were studied. M1δ (3, 8) is a putative transmembrane segment (15), whereas the extramembranous synaptic domain of the α subunits contains a hydrophilic segment- the main immunogenic region (MIR) (10). Neither M1δ nor MIR (α) peptides would be expected to be cytolytic, and indeed they show no activity when tested under the same conditions as M2δ (Fig. 4). Figure 2A,B also show that a polyserine 23-mer has no cytolytic activity. This is significant because serines are postulated to line the polar face of M2δ (4, 8) and lack of cytolysis by polyserine supports the notion that amphiphilicity is a hallmark of channel forming α-helices (7, 8).

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Molecular Engineering of Channel Proteins

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INTRODUCTION AND SCOPE

A central goal in membrane biology is to understand how channel proteins work in terms of their underlying protein structures. Ionic channels are symmetric (or pseudosymmetric) transmembrane protein assemblies organized around a central aqueous pore. The two key functional elements are the *ionic channel* - the actual polar pathway that permits the selective passage of ions across the low dielectric constant apolar core of the membrane lipid bilayer and the *sensor* - the structure that detects the stimulus and couples it to the opening or closing (gating) of the channel. The current excitement in membrane protein science emerges precisely from structural information that is providing clues about the molecular determinants of function. Powerful and sensitive techniques have been developed and are now intensely used to tackle questions about structure-function relationships (cf. 60,74). Molecular cloning and sequencing led to the elucidation of the primary structures of several multi-member gene families (cf. 4,8,36,43,68,74); channel proteins have been purified and reconstituted in lipid bilayers with full retention of function (cf. 62); the properties of many channel proteins have been characterized at the single channel level (83); cDNA or RNA transcripts have been expressed in oocytes as functional proteins (5,32,54,74); molecular modeling has predicted specific peptide segments to form the channel lining that when synthesized by solid phase methods proved to be indeed channel formers in lipid bilayers (77-79). This latter approach - *molecular engineering* - (66) will be reviewed briefly with particular emphasis on the progress we have made in designing peptides that emulate the pore structure of two prototype proteins of two major gene families in the brain, namely the voltage sensitive sodium channel and the nicotinic cholinergic receptor (77-79).

Figure 1 illustrates in the form of a flow chart, the strategy towards the molecular dissection of functional determinants in channel proteins (66).

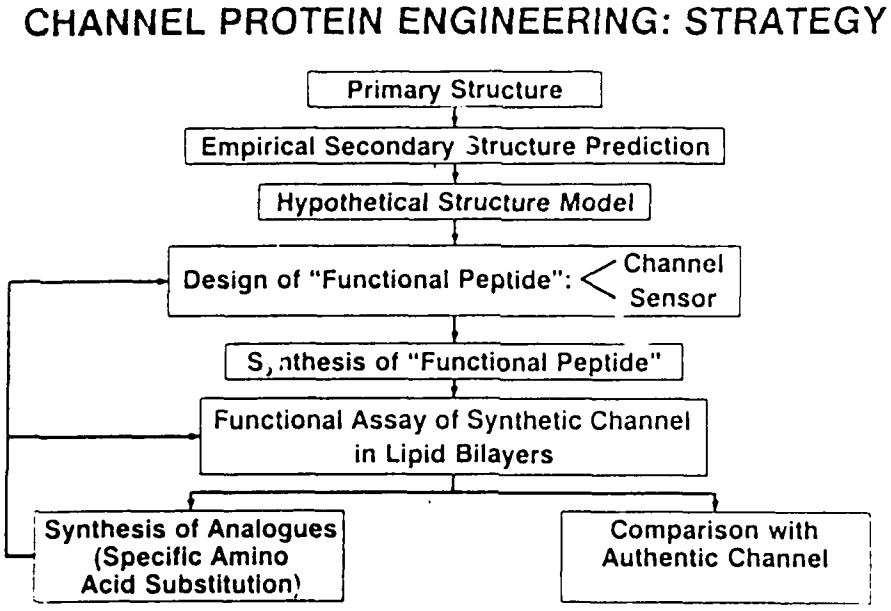

FIG. 1. Channel Protein Engineering: The Strategy.

Given the amino acid sequence for a channel protein, the next step consists of applying empirical secondary structure predictors in order to postulate a structural model of the protein. Secondary structure prediction methods (16,21,28,30,50,56) are empirical and, therefore, are inherently uncertain. Their value resides in their use as a guide to the design and interpretation of experiments. To demonstrate the existence of such functional segments is to design a "functional peptide" that would mimic the predicted structural element: the "channel segment" or the "sensor". To test directly this prediction the functional peptide is synthesized by solid phase methods (3,58,59,92) and its ability to form ion channels is tested by incorporating the peptide into a synthetic lipid bilayer (63,89). The channel activity of the peptide is characterized in detail in the lipid bilayer in terms of ion conduction and channel gating (77-79). This characterization is compared with the specific features of the authentic channel which, in turn, assist in the redesign of the peptide to match the anticipated characteristics of the

authentic channel (66). In addition, the identification of a specific residue thought to be critical for the function under study can then be substituted for an inert one without altering secondary structure in a drastic way. Such analogues provide valuable information to assign functional significance to specific residues (66). The consistency in the structure function relationships is the measure of the reliability of this strategy. This is basically the philosophy behind this approach: the hypothesis is formulated, and the consequences are evaluated experimentally. If any of the predictions is not fulfilled, the model is readjusted and reevaluated.

A remarkable feature inferred from the amino acid sequences of channel proteins is the high homology conservation among members of this family of proteins. Furthermore, protein segments that could be organized as amphipathic transmembrane α-helices are identifiable in all the channel proteins for which sequence information is available (4,8,31,36,68,74,87). This is significant because amphipathic α-helices are structural entities that in a membrane milieu may self-assemble into oligomers. In addition, image analysis of electron micrographs obtained from ordered arrays of channel proteins in their native membrane environment produced low resolution images (approximately 18 Å) that are consistent with symmetric or pseudosymmetric protein assemblies organized around a central aqueous pore (96,97). These key features raise the notion that a unifying motif in the biological design of ion channels is an oligomeric array of transmembrane amphipathic α-helices arranged such that polar residues face a central hydrophilic pore and apolar residues face the hydrophobic bilayer interior (12,21,23,25-27,30,33,34,67). Such structure accounts for the geometric and symmetry constrains of channel proteins, offers a rationale for the extensive homology conservation and provides a basis for the diversity of channel proteins in so far as this would be determined by sequence specificity and oligomer size.

It is worthy of note that several peptides, both of natural origin and synthetic, form channels in lipid bilayers (for reviews see 7,94). Among the natural peptides, the 26-residue peptides melittin (22,95) and *Staphylococcus* aureus δ-toxin (22,57,93) have been studied in detail both from the structural as well as from the channel action points of view. The synthetic channel peptides characterized range from relatively simple sequences containing only leucine and serine residues, for example $(LSSLLSL)_3$ and $(LSLLLSL)_3$ (52) or leucine, serine and glycine $(LSLG)_6$ (45) to more complex, for example, GFLLMITLLILFSQFFLPMILR, a peptide that emulates a segment (residue 16 to 37) of subunit 8 of the H^+-ATP-synthetase of yeast mitochondria (61). A common structural feature of these polypeptides is that they can adopt an amphipathic helical conformation in the bilayer nonpolar interior. If they aggregate, they would have a tendency to form clusters with a central pore involving the polar faces of the helices (e.g. 52,57). This raises the notion that amphipathic peptides of

length sufficient to traverse the width of the bilayer hydrophobic core and organized as bundles of α-helices provide a structural basis for ion-conducting channels (12,21,23,30,33,34).

THE VOLTAGE SENSITIVE SODIUM CHANNEL

Given the primary structure of the sodium channel proteins, several proposals were suggested concerning the folding of the α polypeptide chain across the bilayer membrane (30,34,48,68,71,73,74). We proposed a model for the folding of the polypeptide chain of the sodium channel within the lipid bilayer (30). It consists of four homologous regions, each containing 8 membrane-spanning structures, probably α-helical. The tertiary structure is pseudoradially symmetric. The model suggests the existence of four amphipathic transmembrane helices (1 α-helix contributed by each one of the four homologous repeats) which meet with their hydrophilic faces inward to form a putative ion channel. The central channel created by the boundary of the four helices would be about 4.2 Å across its narrowest dimension, thus accounting for the effective cut off size of the sodium channel (37).

The amino acid sequences of segments postulated to be involved in channel lining for rat brain I, rat brain II (69,70,88), rat brain IIA (2), rat brain III (44,91), E. *electricus* (71), and *Drosophila* sodium channels (84,85) show high degree of homology conservation. We focus here on the structure and results obtained with a synthetic channel peptide with sequence DPWNWLDFTVITFAYVTEFVDL which corresponds to the rat brain I channel segment of homologous repeat I (referred to as Sc1 (30)). Secondary structure predictors suggest that the main structural feature of the synthetic peptide is an α-helix (16,19,28,82,86). Since the spacing between residues is 1.5 Å in an α-helix, such a segment would be about 33 Å long, sufficient to traverse the hydrocarbon core of the membrane (9,30). Circular dichroism spectra of the Sc1 peptide in trifluorethanol show the characteristic features of α-helices, namely, the double minima at 208 and 222 nm and the maximum at 195 nm (14,90). As reported, the synthetic amphipathic 22-mer peptide Sc1, does indeed form transmembrane ionic channels in lipid bilayers (77,78). This is illustrated in Figure 2. The single channel conductance, γ, recorded in symmetric 0.5 M NaCl at 100 mV, is 25 pS for the authentic brain sodium channel (35) and 20 pS for the synthetic channel peptide (77,78). *Similar γ values are reported for the authentic electric eel* (80) and rabbit skeletal muscle (24) sodium channels.

It is clear, that the synthetic channel peptide reproduces several features which are characteristic of the authentic brain sodium channel, such as the similarity in γ, and transitions between the closed and the open state in the millisecond time range (Fig. 2 and Table 1). In contrast, the synthetic channel

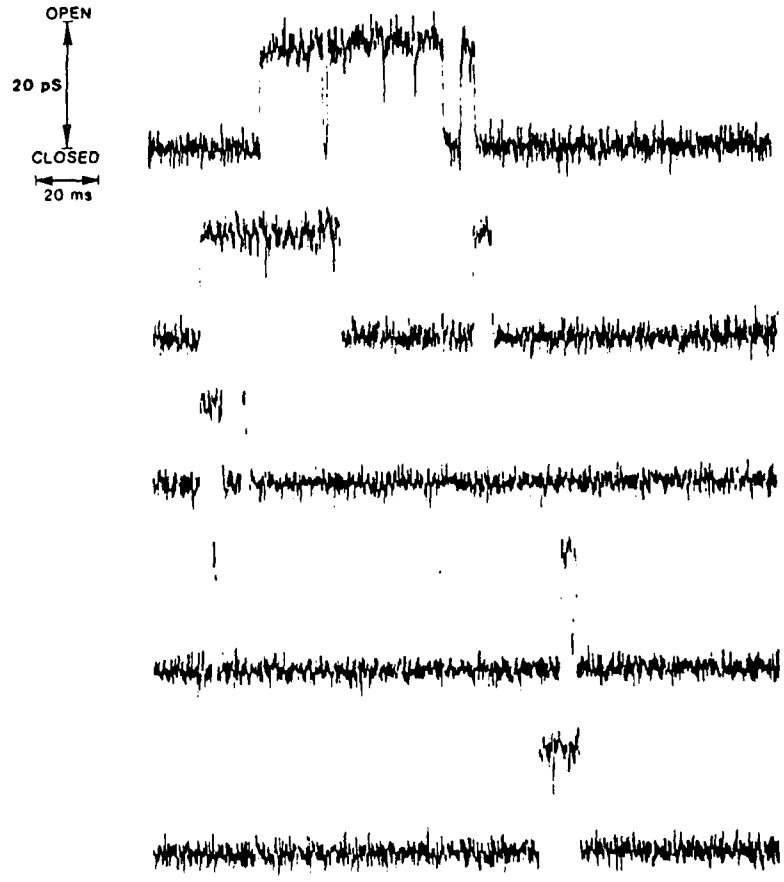

FIG. 2. Single channel currents of the synthetic 22-mer peptide, with the sequence of the rat brain I sodium channel Sc1 segment incorporated in lipid bilayers. Lipid bilayers were formed from diphytanoyl phosphatidylcholine at the tip of patch pipets (63,89) in symmetric 0.5 M NaCl, 5 mM EDTA, 5 mM Hepes pH 7.2. The applied voltage was 100 mV and the records were low-pass filtered at 3 kHz (modified with permission from ref. 78).

peptide differs from the authentic brain channel in lacking discrimination between Na^+ and K^+ ions as well as being insensitive to the applied electric field (39). This disparity was anticipated from the model which considers that the pore is conformed of four distinct amphipathic helices corresponding to the specific chemical sequences of each of the four repeats, and because the voltage sensor is assigned to other distinct transmembrane segments of the array (30).

Model of a Plausible Channel Structure

Energetic considerations suggest that a bundle of four amphipathic α-helices is a plausible channel structure. This emerged from a theoretical search of stable low-energy conformations for the tetrameric α-helical bundles (98) performed by empirical potential energy functions and energy minimizations routines (10,15,47). Figure 3 shows a computer-generated molecular model of the pore-forming structure produced by a bundle of four parallel and aligned α-helices (78). The interior of the structure has an excess of negatively charged residues (D and E) that provide a conductive pathway for cations which is adequate to span the non-polar interior of the bilayer as the hydrophobic residues located on the exterior of the array provide favorable and extensive lipophilic boundaries. The sodium channel selectivity, as postulated (30), appears to result from the location of the

TABLE 1. Ionic Conduction Characteristics of Authentic AChR and Sodium Channel Proteins and of Corresponding Synthetic Channel Peptides

Property	Torpedo AChR[a]	M2δ Peptide[b]	Sodium Channel[c]	Sc1-Peptide[d]
γ_{Na^+} (pS)	45	40	25	20
$\gamma_{K^+}/\gamma_{Na^+}$	1.1	1.2	0.13	1.0
$\gamma_{Tris^+}/\gamma_{Na^+}$	0.18	0.18	N.D.	N.D.
$\gamma_{Cl^-}/\gamma_{Na^+}$	≤0.01	≤0.03	<0.01	≤0.1
Pore Area (Å²)	44[e]	46	16[e]	16
Oligomeric Structure	5-mer	5-mer	4-mer	4-mer

N.D., not determined

[a]Determined in symmetric 0.5 M NaCl, KCl, TrisCl and from reversal potential measurements of *Torpedo* AChR (51,65; and unpublished results).

[b]Refers to the most frequent event determined in symmetric 0.5 M NaCl, KCl or Tris-Cl and from reversal potential measurements (79).

[c]Determined in symmetric 0.5 M NaCl and KCl and from reversal potential measurements of batrachotoxin-modified sodium channels from rat brain (35,49,46).

[d]Refers to the most frequent event, determined in symmetric 0.5 M NaCl and KCl and from reversal potential measurements (78).

[e]37; for review, please see 38.
(Modified from ref. 79.)

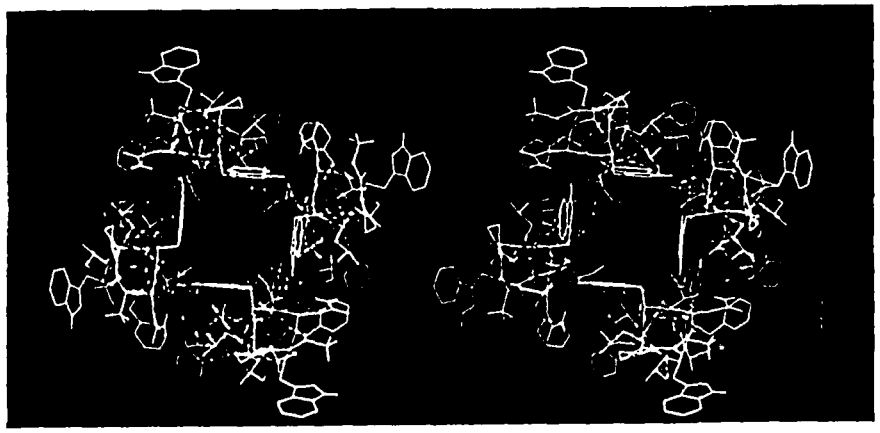

FIG. 3. A bundle of 4 amphipathic α-helices is a plausible structure for the synthetic channel peptide Sc1. Stereo, end-view of a computer generated molecular model of the pore-forming structure. Orthographic projection of the pore showing the four α-helices (blue), the acidic (red), polar-neutral (yellow) and lipophilic (purple) residues. The dimension of the central pore is 4 Å X 4 Å (reproduced with permission from ref. 78).

residues in the channel lining. The effective pore area of the four α-helical cluster is in agreement with the apparent dimension of the authentic sodium channel selectivity filter of 16 Å² (Table 1) (for reviews see 6,11,38). Thus, the tetrameric bundle appears to account for the geometric constrains of the sodium channel.

THE NICOTINIC ACETYLCHOLINE RECEPTOR

The nicotinic acetylcholine receptor (AcChoR) of Torpedo californica is composed of four glycoprotein subunits (α,β,γ,δ) with stoichiometry $\alpha_2\beta\gamma\delta$ 81). A high degree of amino acid sequence homology exists among the four subunits, and all exhibit four putative transmembrane regions designated as M1, M2, M3 and M4 (36,72,74). In vivo, the AcChoR pentamer acts as a ligand activated cation channel (cf. 38) with an effective pore diameter of approximately 7 Å (1,18). The specific assignment of subunits involved in channel lining has been a subject of intense investigation. Affinity labeling with non- competitive channel blockers, such as chlorpromazine (13,29) and triphenylmethylphosphonium (33,40,75,76), identified serine 262 in M2 of the δ subunit as a reactive site. Analysis of the single-channel conductance of AChRs, expressed in *Xenopus* oocytes containing chimeric *Torpedo-bovine* δ

subunits demonstrated that M2 and the adjacent segment connecting M2 with M3 exert a profound effect on ion conduction through open AChR channels (41). These observations provided the clue that M2 was a candidate to be involved in forming the receptor pore structure (for recent reviews see 17,60).

Figure 4 shows that the synthetic peptide with a sequence that emulates that of M2δ of the Torpedo AChR (EKMSTAISVLLAQAVFLLLTSQR) indeed forms discrete channels in lipid bilayers (79). Single channel conductance histograms indicate that γ in symmetric 0.5 M NaCl = 39 pS. The conductance selectivity ratio is Na^+:K^+:$Tris^+$ = 1.0:1.2:0.18. This selectivity sequence is comparable to that characteristic of the authentic AChR channel (Table 1).

It is evident that this synthetic peptide mimics some features which are characteristic of the AChR channel (Table 1): γ, cation selectivity, and channel lifetimes for both open and closed states in the millisecond time range (79). The peptide, however, does not reproduce the cholinergic ligand-dependent behavior of the native channel, nor should it be expected to do so. However, its ability to form discrete channels supports the plausibility that this segment of the protein may conform the ion conductive pathway of the AChR channel.

A distinct feature of the single channel recordings obtained with both synthetic channel peptides (Table 1), is the occurrence of opening events with distinct γ amplitudes and variable open and closed lifetimes (78,79). Thus far, our description focused on the most frequent conductance events (Table 1) with γ = 20 pS and γ = 40 pS for Sc1 and M2δ, respectively (78,79). However, smaller (γ ≤ 10 pS) and larger (γ ≥ 60 pS) events are detected at significantly lower frequency of occurrence. This is illustrated in Figure 4 where the occurrence of distinct events with γ = 20 pS and γ = 40 pS

FIG. 4. Single channel currents of the synthetic 23-mer peptide, with the sequence of the Torpedo M2δ incorporated in lipid bilayers. Lipid bilayers were formed from diphytanoyl-phosphatidylcholine at the tip of patch pipets (63,89) in symmetric 0.5 M NaCl, 5 mM Hepes, pH 7.2. The synthetic peptide was dissolved in trifluorethanol (Aldrich, Milwaukee, WI) and added to the aqueous phase, bathing the bilayer to a final concentration of 0.1 μg/ml. The records were obtained several minutes after peptide addition. The applied voltage was 100 mV and the records were low-pass filtered at 2 kHz (Myrta Montal and Mauricio Montal, unpublished results).

pS for the M2δ peptide is clearly discerned. In general, smaller conductances show larger τ_o whereas larger events appear in brief bursts of openings (78,79). This heterogeneity of γ and τ_o suggests that the channel recorded in bilayers is the expression of a non-covalently bonded oligomer that self-assembles in the membrane to acquire a minimum energy configuration. In keeping with the AChR subunit stoichiometry and sequence data, and noting the similar g of the synthetic peptide and the purified authentic AChR channel, γ = 45 pS) (64) under identical recording conditions, it was suggested that the recorded channel, with γ = 40 pS, arises from a bundle of five parallel α-helices. Indeed, circular dichroism spectra of the M2δ peptide in trifluorethanol showed that the predominant secondary structure of the peptide was α-helical. From the CD intensity at 222 nm the helical content was estimated to be ~ 60% (90). Considering an interaxial helical distance of 9 Å, as inferred from crystallographic data of soluble proteins that exhibit a bundle structure of α-helices (98,56), a pore area of 44 $Å^2$ is calculated for a pentameric array (Fig. 5). Such value is consistent with the estimated AChR pore size as inferred from electrophysiological measurements (1,18,38,55; see Table 1).

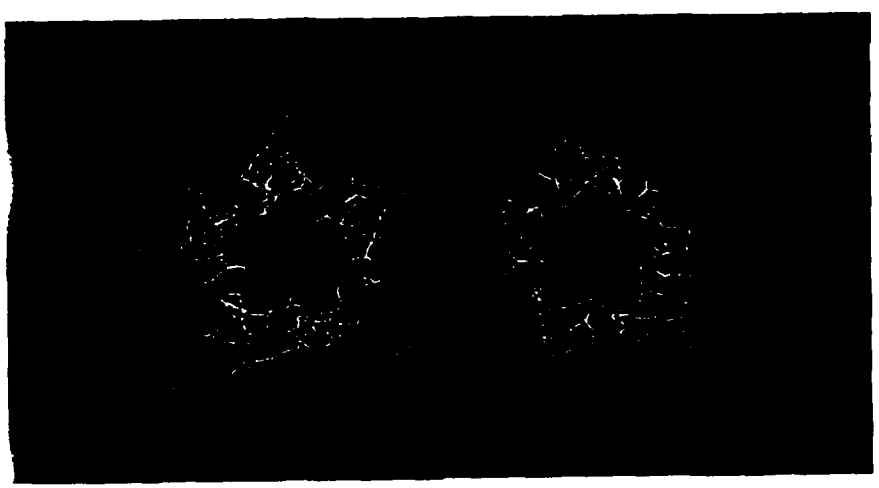

FIG. 5. Stereo, end-view of pentameric C_5-symmetric array of the 23-mer synthetic M2δ peptide. The N-terminus is in the front and is assigned to the cytoplasmic face of the membrane. The α-carbon backbone of the five helices is shown in light blue. Amino acid sidechains are colored by functional type: basic, blue; acidic, red; polar-neutral, orange; and lipophilic, violet. The lowest energy structure shown has S8 facing the lumen of the pore. The area of the central pore at its widest extent is 46 $Å^2$. (Reproduced with permission from ref. 79).

Model of a Plausible Channel Structure

The pentameric parallel helical array of the model channel has an internal pore with a diameter ranging from approximately 4 Å at its narrowest point to approximately 6 Å at its widest extent. Nonpolar residues are predominantly on the outside and polar residues predominantly on the inside, except at the two ends of the helices. Residues S4, T5, S8, A12, F16 and T20 face the lumen of the pore. The R23 side chains bridge the C-termini of neighboring helices forming a ring at one end of the pore. At the other end, E1 bridges the amino groups of the N-terminus and K2 within each helix. This symmetric pentamer conforms to the general features of the packing arrangement postulated for the $\alpha_2\beta\gamma\delta$ AChR pentamer: The outside is lipophilic, the inside more hydrophilic, and S8 (corresponding to S262 of the AChR δ subunit) is exposed on the channel lumen in accord with chemical labeling experiments.

Recently, AChRs with mutations at various sites in the M2 segment, were expressed in *Xenopus* oocytes after injection of cloned mRNAs and the single channel properties analyzed (42,53). Focusing on M2δ a change in E1 (E 255) for Q produced AChRs with a drastic reduction in g (2 fold) and in the sensitivity of outward and inward channel currents

to intracellular and extracellular $[Mg^{2+}]$, respectively (42). The $\alpha_2\beta\gamma\delta$ stoichiometry of AChRs enables the addition or deletion of serine residues at the conserved position corresponding to S8 in the M2δ-mimicking peptide yielding mutant AChRs with 0,1,2,3 or 4 serine residues at this site. The binding affinity of mutant AChRs for the QX-222 channel blocker increased with the number of serine residues (53). This result is in gratifying accord with affinity labeling and sequencing results (29,76), the expectation of residues containing OH as contributors to the structure lining the channel of a water-filled pore (cf. 20), the transmembrane arrangement of M2 (42) and the channel activity of the M2δ mimicking peptide (79).

An issue that must be addressed concerns the specificity of the channel formation by synthetic peptides. All models postulate (e.g. 21,33,36,74) very hydrophobic transmembrane segments with an excess of non-polar residues such as V,I,L,F and A as components of the array. These segments occur at the interface between homologous regions or between subunits providing favorable and extensive hydrophobic boundaries. For the sodium channel rat brain I the sequence of such a segment, Sh1, is: IFFVLVIFLGSFYLIN LILAVV (30). The synthetic Sh1 peptide was incorporated into lipid bilayers and, indeed, it does not form channels (Oiki, S., Danho, W. and Montal, M., unpublished results).

For the *Torpedo* AChR the sequence of such a segment, M1δ, is: PLFYVINFITPCVLISFLASLAFYLPA. This peptide does not form channels (79). These results support the reliability of the approach.

The results and interpretations outlined above for both the voltage

sensitive sodium channel and the AChR synthetic channel peptides represent a starting point upon which to build up the structure of the authentic channel. The ionic conduction characteristics of authentic *Torpedo* AChR and rat brain sodium channel proteins and of the corresponding synthetic channel peptides, M2δ and Sc1, respectively, are compared in Table 1 (78,79). The peptide that mimics the AChR M2δ-segment forms channels in bilayers with properties remarkably similar to those of the authentic protein. In contrast, the sodium channel peptide mimetic lacks the selectivity between Na^+ and K^+ that is so distinctive of the authentic channel as well as its exquisite regulation by voltage (39). Therefore, *selectivity* and *gating* are our immediate aims and understanding their origin our guide.

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ABBREVIATIONS USED

AChR, acetylcholine receptor; ACh, acetylcholine; V, applied voltage; γ, single channel conductance; τ_o, open channel lifetime.

When referring to specific amino acids, standard one-letter codes are used: A, alanine; R, arginine; N, asparagine; D, aspartate; C, cysteine; E, glutamate; Q, glutamine; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; V, valine.

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387.3

EXPRESSION OF RAT MUSCLE ACETYLCHOLINE RECEPTOR EPSILON SUBUNIT IN XENOPUS OOCYTES. P. Camacho, R. H. Chapman*, G. Mandel*, and P. Brehm. Dept. of Physiology., Tufts Univ. Med. Sch., Boston, MA 02111 and Div. Mol. Med., New England Med. Ctr., Boston, MA 02111.

The nicotinic acetylcholine receptor channel from muscle is a pentameric protein assembled from alpha, beta, gamma, and delta subunits. A fifth subunit, epsilon, has been cloned from calf and more recently, from rat (Criado et al., Nucleic Acids Res. 16,1933 and Camacho et al., Biophysics J. 55,1989). Using the Xenopus oocyte translation system, we have studied the single channel properties of acetylcholine receptor channels expressed from synthetic mRNA transcripts. We have obtained functional expression of AChR channels by injecting alpha, beta and delta from mouse, in conjunction with mRNA transcripts from rat epsilon subunit. Injection of mRNAs encoding alpha, beta, delta, epsilon subunits results in inward currents of several microamperes at the macroscopic level. Single channel recordings from outside-out patches revealed two amplitude classes of ACh-activated channels. Both had briefer open times and higher conductances than those obtained by alpha, beta, gamma and delta. Injection of alpha, beta and gamma subunit RNAs result in the expression of substantial macroscopic currents. However, in one experiment injection of alpha, beta and epsilon transcripts did not express functional channels, suggesting that receptors containing epsilon may be conditional on the presence of delta subunit.

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387.4

SYNTHESIS OF A CHANNEL PROTEIN AND CHARACTERIZATION OF ITS SINGLE CHANNEL PROPERTIES. M. Montal, M.S. Montal* and J. M. Tomich*. UCSD, La Jolla, CA 92093 and Children's Hospital, Los Angeles, CA 90027

A 23-mer peptide with the sequence of the M2 segment of the *Torpedo* acetylcholine receptor δ-subunit (EKMSTAISVLLAQAVFLLLTSQR) forms cation-selective channels in lipid bilayers (*PNAS* 85:3703-3707). Channel formation presumably involves self-assembly of conductive oligomers. Here, we synthesized a tethered parallel tetramer with M2δ peptides attached to a multifunctional carrier template (*Tetrahedron* 44:771-785): a 9-aminoacid backbone K*KK*PGK*EK*G with K containing N^{α}-tboc, N^{ϵ}-fmoc(*) to generate 4 branch points. M2δ was then attached to template in a stepwise manner at the 4 base-deprotected K side chains. The complete 101 residue protein was cleaved in HF and purified by RP-HPLC. It migrates as a single band in SDS-PAGE (15%) with apparent M_r 11 000. The synthetic channel protein does indeed form channels in phosphatidyl choline bilayers. The single channel conductance γ, channel open (τ_o) and closed (τ_c) lifetimes and percent open time (Po) in symmetric 0.5M NaCl or KCl (10 mM Hepes, 0.5mM $CaCl_2$, pH 7.5) at 100 mV are:

NaCl- γ=18 pS; τ_{o1}=0.3 ms, τ_{o2}=25 ms; τ_{c1}=1.0 ms, τ_{c2}= 17 ms; Po=66%.
KCl- γ=24 pS; τ_{o1}=1.7ms, τ_{o2}=11 ms; τ_{c1}=0.9 ms, τ_{c2}= 4.5 ms; Po=47%.

Membrane conductance increased in discrete steps, integral multiples of these elementary conductances, indicating that the tethered tetramer is the conductive species. We conclude that a four-helix bundle protein is a plausible structure underlying the conductance events.

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387.5

SINGLE-CHANNEL CURRENTS ACTIVATED BY GABA, MUSCIMOL AND ISOGUVACINE IN OUTSIDE-OUT MEMBRANE PATCHES FROM CULTURED CHICK CEREBRAL NEURONS. [illegible] and [illegible]. Neurobiology Res. Ctr., Univ. of Al. at Birmingham.

Single-channel currents activated by GABA (500nM-1mM) muscimol (100-500nM) and isoguvacine (500nM-1mM) were recorded from outside-out patches in isotonic Tris-Cl solutions. Under these conditions GABA-activated channels had multiple conductance states of 12, 19, 26 and 31 pS. Transitions between this and the other conductance levels were found. Muscimol and isoguvacine activated similar conductance levels. For all agonists, the main conductance level was around 26 pS. I-V relations for all agonists were linear and reversed around 0 mV. Analysis of the main-state openings gated by GABA revealed two time constants of 0.13 ± 0.04 and 2.37 ± 0.17 ms (n=3). Similar time constants of 0.43 ± 0.10 and 2.21 ± 0.40 ms (n=4) were obtained for isoguvacine. Open time distributions for muscimol were best described by the sum of three exponentials having time constants of 0.16 ± 0.08, 1.15 ± 0.41 and 5.10 ± 4.07 ms. GABA, muscimol and isoguvacine activate similar multiple conductance levels in chick cerebral neurons. Opentime kinetics for GABA and isoguvacine are similar while muscimol openings displayed an additional longer open state. Additionally, in some patches the occurrence of subconductance levels were more frequent with muscimol and isoguvacine. Supported by NS11535, NS13145 and NS22373.

387.6

TWO NOVEL $GABA_A$ RECEPTOR SUBUNITS EXIST IN DISTINCT NEURONAL SUBPOPULATIONS. B.D. Shivers, I. Killisch*, R. Sprengel*, H. Sontheimer*, M. Köhler*, P.R. Schofield and P.H. Seeburg. Center for Molecular Biology, NF 282 and *Institute for Neurobiology, NF 364, University of Heidelberg, D-6900 Heidelberg, F.R.G.

Two cDNAs encoding novel $GABA_A$ receptor subunits were isolated from a rat brain library. These subunits, named γ2 and δ, share approximately 35% sequence identity with α and β subunits and, like these, form functional GABA-gated chloride channels when expressed alone in cultured cells. The γ2 subunit represents the rat homolog of the recently described human γ2 subunit shown to be important for benzodiazepine (BZ) pharmacology (D.B. Pritchett et al., Nature, 338:582, 1989). Cellular localization of the mRNAs encoding the γ2 and δ subunits in rat brain revealed that largely distinct neuronal subpopulations express the two subunits. The distribution of the δ subunit resembles that of high affinity $GABA_A$ receptors labeled by ^{3}H-muscimol while the localization of the γ2 subunit resembles that of $GABA_A$ BZ receptors labeled with ^{3}H-flunitrazepam. These findings have implications for the subunit composition of two different $GABA_A$ receptor subtypes and for information processing in neural networks using GABA for signaling.

387.7

HUMAN $GABA_A$ RECEPTORS ASSEMBLED FROM DIFFERENT COMBINATIONS OF CLONED SUBUNITS HAVE DIFFERENT ELECTROPHYSIOLOGICAL PROPERTIES. Todd A. Verdoorn, Andreas Draguhn*, Bert Sakmann*, Dolan B. Pritchett, Peter H. Seeburg*. Max-Planck-Institut für medizinische Forschung and ZMBH, Heidelberg, FRG

Cultured human embryonic kidney cells were transiently transfected with cDNA clones encoding α_1, β_1, and γ_2 subunits of the human $GABA_A$ receptor. Chloride currents activated by GABA were examined using patch clamp in the whole cell and outside-out configurations (symmetrical Cl-, 140 mM). In cells transfected with cDNAs encoding the α_1 and β_1 subunits whole cell currents (-60 mV) elicited by fast application of 10 μM GABA desensitized markedly to 16 ± 4% (mean ± SD, n=4) of the initial peak current after 10 sec. In contrast, cells transfected with cDNAs encoding the α_1, β_1, and γ_2 subunits (74 ± 18 % of peak after 10 sec., n=4, 10 μM GABA) or only α_1 and γ_2 (no detectable desensitization over 10 sec., n=5, 30 μM GABA) showed much less desensitization. Single channel GABA-activated currents in outside-out patches (-50 mV) from cells transfected with DNAs encoding α_1 and β_1 subunits had a mean conductance of 19.9 ± 1.2 pS (n=4, 10 μM GABA). The single channel conductance was higher in cells cotransfected with DNA encoding the γ_2 subunit: α_1, β_1, and γ_2 transfected together yielded a mean single channel conductance of 36.4 ± 2.5 pS (n=4, 10 μM GABA); cells transfected with α_1 and γ_2 showed a mean conductance of 34.5 ± 2.5 pS (n=3, 30 μM GABA). The reduced desensitization and higher single channel conductance upon cotransfection with DNA encoding the γ_2 subunit may result from the formation of a new $GABA_A$ receptor subtype or the expression of a mixture of subtypes.

387.8

PRIMARY CULTURES OF MOUSE SPINAL CORD EXPRESS THE NEONATAL ISOFORM OF THE INHIBITORY GLYCINE RECEPTOR. W. Hoch*, H. Betz and C.-M. Becker*. ZMBH, Universität Heidelberg, Im Neuenheimer Feld 282, D-6900 Heidelberg, FRG.

Glycine receptor expressed by primary cultures of spinal cord is predominantly of the recently identified neonatal isoform characterized by a low affinity for strychnine. Its ligand binding subunit differs from that of adult receptor in antigenic epitopes and molecular weight. Whereas in vivo the neonatal receptor isoform is completely replaced by the adult isoform within three weeks after birth, this exchange of subtypes is not seen in culture. However, the increased expression of the cytoplasmic glycine receptor-associated 93 kD protein occurring after birth is also observed in culture. Purification of glycine receptor from cultures yielded polypeptides of 49 kD and 93 kD suggesting that the membrane-spanning core of the neonatal receptor may be a homooligomer composed of 49 kD subunits. Pulse-labeling experiments revealed the 49 kD subunit to be a metabolically stable glycoprotein (half life =2 days).

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